Enhanced soluble sugar content in tomato fruit by CRISPR/Cas9-mediated two genes editing (#58708)

First submission

Guidance from your Editor

Please submit by **26 Mar 2021** for the benefit of the authors (and your $200 publishing discount) .

Structure and Criteria
Please read the 'Structure and Criteria' page for general guidance.

Raw data check
Review the raw data.

Image check
Check that figures and images have not been inappropriately manipulated.

Privacy reminder: If uploading an annotated PDF, remove identifiable information to remain anonymous.

Files

Download and review all files from the materials page.

5 Figure file(s)
1 Table file(s)
1 Raw data file(s)

Structure and Criteria

Structure your review

The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

When ready submit online.

Editorial Criteria

Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Speculation is welcome, but should be identified as such.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult. I suggest you have a colleague who is proficient in English and familiar with the subject matter review your manuscript, or contact a professional editing service.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Enhanced soluble sugar content in tomato fruit by CRISPR/Cas9-mediated two genes editing

Baike Wang [Corresp., 1], **Ning Li** [1, 2], **Shaoyong Huang** [2], **Jiahui Hu** [1, 3], **Qiang Wang** [1, 2], **Yaping Tang** [1], **Tao Yang** [1], **Patiguli Asmutola** [1], **Juan Wang** [1], **Qinghui Yu** [1]

[1] Institute of Horticulture Crops, Xinjiang Academy of Agricultural Sciences, Urumqi, China

[2] College of Forestry and Horticulture, Xinjiang Agricultural University, Urumqi, China

[3] Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Urumqi, China

Corresponding Author: Baike Wang
Email address: 46367247@qq.com

Due to the importance of soluble sugar in improving the flavor and increasing the yield of tomato sauce, several studies have focused on improving the content of soluble sugar in tomato fruits. However, previous studies commonly increased the soluble sugar content by promoting the functional genes or activating the key protein kinases. In this study, two genes (*SlINVINH1* and *SlVPE5*) that inhibited the accumulation of soluble sugar in tomato fruits were identified. We obtained the knocked-out lines of two genes by CRISPR/Cas9, respectively. Then, the aggregated lines with both CRISPR-*invinh1* and CRISPR-*vpe5* were gained by hybridization and self-pollinating. Compared to the wild-type lines, the glucose, fructose and total soluble solids (TSS) contents of CRISPR-*invinh1* or CRISPR-*vpe5* were significantly increased. In addition, the levels of glucose, fructose and TSS showed a further improved in the lines with CRISPR-*invinh1* and CRISPR-*vpe5* simultaneously than that of the single gene knock-out lines, which indicated that the two genes had a synergistic effect in increasing the content of these soluble sugars. Thus, knock-out the *SlINVINH1* and *SlVPE5* could effectively increase the content of soluble sugars and might provides an important theoretical guidance and practical basis for improving the flavor of tomato fruits and processing quality.

Enhanced soluble sugar content in tomato fruit by CRISPR/Cas9-mediated two genes editing

Baike Wang[1], Ning Li[1,2], Shaoyong Huang[2], Jiahui Hu[1,3], Qiang Wang[1,2], Yaping Tang[1], Tao Yang[1], Patiguli Asmutola[1], Juan Wang[1,*], Qinghui Yu[1,**]

[1] Institute of Horticulture Crops, Xinjiang Academy of Agricultural Sciences, Urumqi, China, [2] College of Forestry and Horticulture, Xinjiang Agricultural University, Urumqi, China, [3] Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi, China

*Corresponding author: Juan Wang, E-mail: 278089648@qq.com

**Corresponding author: Qinghui Yu, E-mail: yuqinghui98@sina.com

Running title: Enhanced soluble sugar content in tomato fruit

ABSTRACT

Due to the importance of soluble sugar in improving the flavor and increasing the yield of tomato sauce, several studies have focused on improving the content of soluble sugar in tomato fruits. However, previous studies commonly increased the soluble sugar content by promoting the functional genes or activating the key protein kinases. In this study, two genes (*SlINVINH1* and *SlVPE5*) that inhibited the accumulation of soluble sugar in tomato fruits were identified. We obtained the knocked-out lines of two genes by CRISPR/Cas9, respectively. Then, the aggregated lines with both CRISPR-*invinh1* and CRISPR-*vpe5* were gained by hybridization and self-pollinating. Compared to the wild-type lines, the glucose, fructose and total soluble solids (TSS) contents of CRISPR-*invinh1* or CRISPR-*vpe5* were significantly increased. In addition, the levels of glucose, fructose and TSS showed a further improved in the lines with CRISPR-*invinh1* and CRISPR-*vpe5* simultaneously than that of the single gene knock-out lines, which indicated that the two genes had a synergistic effect in increasing the content of these soluble sugars. Thus, knock-out the *SlINVINH1* and *SlVPE5* could effectively increase the content of soluble sugars and might provides an important theoretical guidance and practical basis for improving the flavor of tomato fruits and processing quality.

Keywords: soluble sugars, CRISPR/Cas9, genome editing, tomato fruits, total soluble solids

51

52 INTRODUCTION

53 Tomato (*Solanum lycopersicum*) is an important vegetable and commercial crop that enjoys great
54 popularity worldwide. However, modern methods for the cultivation of tomatoes have been
55 criticized for unsatisfactory fruit flavors. Sweetness, as an important flavor characteristic of tomato
56 fruits, is mainly determined by the content of soluble sugars in the fruits (Tieman et al., 2017). In
57 addition to influencing the flavor, soluble sugar is also the most important component of the total
58 soluble solids (TSS), which is also a major factor affecting the cost of production of processed
59 tomato and tomato sauce. Studies have reported that while producing tomato sauce at a
60 concentration of 28%, an increase in the TSS content of processed tomatoes from 4% to 5% could
61 reduce the raw material consumption by up to 25% (Gur & Zamir, 2015). Therefore, increasing
62 the content of soluble sugar in tomatoes can not only improve the flavor quality of fresh tomatoes
63 but also increase the efficiency of the production of tomato sauce.

64 Several factors play roles in influencing the content of soluble sugar in tomato fruits, including
65 genetic factors and environmental factors (temperature, light, moisture, air, fertilizer, plant
66 hormones, etc.) that play a direct role, as well as the technical factors of cultivation and
67 management that play an indirect role through the environmental factors (Beckles, 2012). Among
68 these factors, genetic factors are fundamental, based on which other factors exert their effects.
69 Although some studies have investigated genes related to soluble sugars, the corresponding
70 biological functions of most of these genes are not fully understood (Lupi et al., 2019). Therefore,
71 the immense potential economic value associated with tomatoes makes it necessary to conduct in-
72 depth research and exploration of these genes, and further combine the knowledge with molecular
73 marker-assisted selection or genetic engineering techniques to increase the content of soluble
74 sugars in cultivated tomatoes (Gur & Zamir, 2015; Vallarino et al., 2017). Meanwhile, the
75 investigation of the genes related to soluble sugars also helps in further analysis of the molecular
76 mechanisms of the accumulation of soluble sugars in tomato and provides a reference for
77 understanding the quality of other fruit types, which has substantial theoretical significance.

Plants have a set of complex sugar metabolic mechanisms, including the sensing of sugar signals, sugar synthesis, loading, transportation, unloading, transformation, storage, accumulation, and regulation of several physiological and biochemical processes. Several studies have largely focused on enzymes and proteins related to the processes of sugar synthesis, transportation, and decomposition, including invertase, sucrose synthase (SUS), sucrose phosphate synthase (SPS), hexose kinases (Hexokinase, HXK), fructokinases (FRKs), sucrose phosphatase (Sucrose Phosphate Phosphatase, SPP), sugar transporters, and some transcription factor (Beckles et al., 2012; Patrick, Botha & Birch, 2013). These studies have facilitated a deeper understanding of the molecular mechanisms of sugar accumulation.

Invertase has a significant effect on sugar accumulation. Beets (*Beta vulgaris*) mainly accumulate sucrose, while tomatoes largely accumulate hexose. During the early stage of sugar accumulation in beets, the activity of insoluble acid invertase in the cell wall decreased dramatically, while in tomatoes, the activity of acid invertase increased significantly at the same stage (Patrick, Botha & Birch, 2013). A cloned QTL (Brix9–2–5) that relates to the accumulation of soluble sugar is the *Lin5* gene. The content of soluble sugar in the introgression line (IL) containing this site is significantly higher than in the IL without this site (Baxter et al., 2005), and the specific silencing of *Lin5* reduced the concentration of soluble sugar in the pulp (Zanor et al., 2009). Accumulation of sugar can also be regulated by controlling the metabolic flow. The accumulation of sucrose in sugarcane can generate feedback inhibition. When the conversion of sucrose to maltulose and kestose is promoted by transgenic manipulations, it not only increases the demand for carbon, but also reduces the feedback inhibition of sucrose, increases the rate of photosynthesis of leaves, and elevates the content of total sugars (Wu & Birch, 2007). Manipulation of the genes regulating sucrose isomerase (SI) and ADP-glucose pyrophosphorylase (AGPase) promotes the conversion of sucrose to its isomers and starch and increases the sugar content of transgenic tomatoes (Petreikov et al., 2009). Some transcription factors are also involved in the regulation of the metabolism of fruit sugars. For example, the transcription factor RIN, which is related to fruit maturation, can directly bind to the promoter regions of the vacuolar

105 invertase (*SlVI*) gene and vacuolar invertase inhibitor (*SlVIF*) gene, and regulate their expression,
106 thereby affecting sugar metabolism and maturation (Qin et al., 2016). ABA is essential for the
107 accumulation of sugar in tomato fruits (Li et al., 2019). ABA induces the expression of the NAC
108 transcription factor gene *SlNAP2*, which in turn can regulate the synthesis of ABA and influence
109 tomato senescence, which further influences sugar metabolism. Transgenic tomato with RNA
110 interference exhibited delayed senescence of leaves, increased yield, and higher content of soluble
111 sugar in fruits (Ma et al., 2018). The gene *SlGLK*, which controls the shoulder development of
112 tomato fruits, also affects the accumulation of sugar in the fruit. The overexpression of this gene
113 turns the fruit color into dark green and significantly increases the content of starch, sugar, and
114 TSS, which indicates a potential relationship between increased fruit chlorophyll content and
115 enhanced photosynthetic efficiency (Powell et al., 2012).

116 The gene *INVINH1* (invertase inhibitor) is an inhibitor of the acid invertase gene *Lin5*, which
117 specifically inhibits the activity of cell wall invertase. Studies have reported that the silencing of
118 the invertase inhibitor corresponding to the *Lin5* gene can increase the activity of invertase and
119 significantly increase the content of soluble sugar in the fruit, which in turn increases the TSS
120 content of the fruit (Jin, Ni & Ruan, 2009; Ruan, Jin & Huang, 2009). The gene *SlVPE5* belongs
121 to the family of VPE (vacuolar processing enzyme) genes. *SlVPE5* negatively regulates the
122 accumulation of sugar (Ariizumi et al., 2011; Wang et al., 2016). However, the mechanism by
123 which *INVINH1* and *SlVPE5* negatively regulate sugar accumulation has not been elucidated, and
124 the interactive relationship between *INVINH1* and *SlVPE5* has not been studied before.

125 Therefore, to study and increase the content of soluble sugar in tomato fruits, this study analyzed
126 the structural types and genetic relationship of two genes (*INVINH1* and *SlVPE5*) that could inhibit
127 the accumulation of soluble sugar. Using CRISPR/Cas9 technology, both the genes were knocked-
128 out separately to obtain mutants with the loss of function of each gene, and the influence of each
129 gene on each type of glucose, fructose and TSS during fruit ripening was analyzed. Through
130 hybridization and self-pollinating, the lines with loss of function of both genes was obtained. We
131 have further explained the regulatory relationship between these genes and the accumulation of

soluble sugar, and have provided significant theoretical guidance and practical basis for the improvement of the flavor of tomato fruits and trait processing.

MATERIALS & METHODS

Plant material and growth cnditions

Cultivated tomato M82 was used as the experimental material. All the tomato materials were grown in the greenhouse at the seedling stage (Environmental conditions: 14 h light/10 h dark, the daytime temperature of 25 °C, and night temperature of 18 °C). The seedlings were transplanted to the field before entering the flowering stage, wherein the management of the test field was the same as that of the general field. The testing field was located at the Comprehensive Testing Field (87°47′ E, 43°95′ N) of Xinjiang Academy of Agricultural Sciences in Urumqi, China.

Sample preparation

The genotypes of transgenic seedlings and wild-type seedlings were identified when the plants grew relatively stronger (at least four leaves), and genomic DNA from the leaves was extracted for the detection of mutations. Detection of soluble sugar and TSS content in the fruit was carried out when the fruit was ripe. At least six fruits (two from each replicate with three replications) were collected from the second and third spikes of each plant, crushed, and homogenized into a sauce, and then analyzed for the content of soluble sugar and TSS.

Phylogenetic analysis

The protein sequences of INVINH and VPE from tomato (*Solanum lycopersicum*), *Arabidopsis thaliana*, paper (*Capsicum annuum*), citrus (*Citrus sinensis*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), grape (*Vitis vinifera*), and maize (*Zea mays*), which the BLAST database search analysis were performed at NCBI. Multiple alignments of the INVINH and VPE protein sequences were processed by MEGA7 (Kumar, Stecher & Tamura, 2016). The phylogenetic tree was constructed by the neighbor-joining statistical method using 1000 bootstrap replicates (Kumar, Stecher & Tamura, 2016).

Selection of sgRNA target sequence and CRISPR/Cas9 vector construction

The platform CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) was utilized to design the target sites of the target genes *SlINVINH1* (Solyc12g099200.1) and *SlVPE5* (Solyc12g095910.1). Each gene was provided with two target sites (Fig. 2B). The CRISPR/Cas9 vector was constructed as described previously (Yu et al., 2017). The gene of CRISPR/Cas9 was driving by 35S promoter, sgRNA1 and sgRNA2 were under the control by the U6 promoter of *Aribidopsis thaliana* and tomato, separately. All constructs were assembled using the Circular Polymerase Extension Cloning (CPEC) method (Quan & Tian, 2009). The pCAMBIA1301 binary vector (AtU6-sgRNA1-SlU6-sgRNA2-35S-Cas9) was constructed, which used for genes knock-out (Fig. 2A).

Plant transformation

The Agrobacterium-mediated transformation method (Yu et al., 2017) was used, and pCAMBIA1301 vectors containing the Cas9 and sgRNA cassette were transformed into M82. In brief, tomato seeds were germinated on ½ MS medium after sterilization with 10% NaClO. After 9-12 days culture, the apical segments of hypocotyls were punctured with OD600 = 0.5-0.6 of Agrobacterium suspension. Then, the explants were inoculated on selective plates with hygromycin (3 mg/L) until transgenic plants were regenerated from the calluses. After *in vitro* regeneration, plants were transplanted into soil in light growth chamber.

DNA extraction and mutation detection

Genomic DNA from fresh frozen leaves was extracted using high efficiency plant genome DNA extraction kit (Tiangen, Beijing, China), and the genomic flanks containing the target sites were amplified using specific primers (Table S1). Then, the annealed PCR products were subjected in to 1% agarose. The cut and purified PCR products were cloned into the pZERO-T Vector (Transgen, Beijing, China), and 26 clones for mutagenesis were sequenced using the Sanger method at each plant with the M13 primer. For the genotyping of T_1 and T_2 plants obtained from the T_0 lines by strict self-pollination, the target fragment was directly sequenced. For the genotyping of F_2 plants obtained from the F_1 lines by strict self-pollination, and the F_1 lines obtained from the two T_0 lines cross-pollination, which the target fragments were also directly sequenced.

Determinations of soluble sugars and TSS

Soluble sugars were determined as described previously (Lupi et al., 2019). Glucose, fructose and sucrose were quantified by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex, Sunnyvale, CA, USA) using a Carbopac PA1 column (250 x 4 mm, 5 µm particle size, Dionex) in an isocratic run with 18 mM NaOH as mobile phase. Content of each sugar was calculated using standard curves made with pure glucose, fructose and sucrose. TSS were measured with a refractometer DR201-95 (Kruess, Germany). Each sample contained three replicates with two fruit per replicate.

RESULTS

Characteristics and phylogenetic analysis of genes

The invertase inhibitor (INVINH1) protein consists of 171 amino acid residues, with a signal peptide of 19 amino acid residues at the N-terminus. The differences between INVINH1 and SolyCIF, which is another type of invertase inhibitor in tomato, include distinctions in protein sequences and protein structures (Reca et al., 2008). Compared with the sequence of the INVINH1 protein in other crops, four cysteine residues were conserved, which was also a significant feature of all invertase inhibitors (Rausch & Greiner, 2004). Cluster analysis revealed that the INVINH1 protein of tomato had the closest relationship with that of potato and tobacco, which are also solanaceous crops, but was distantly related to *Arabidopsis*, corn, and rice (Fig. 1A).

The vacuolar processing enzyme (VPE) has been classified in the cysteine protease family and is mainly involved in the regulation of post-translational levels of proteins in vacuoles. The VPE proteins are highly conserved in most plants and animals (Hara-Nishimura, Takeuchi & Nishimura, 1993). Among the numerous VPE proteins in tomatoes, SlVPE3 and SlVPE5 have been reported to regulate the accumulation of soluble sugars (Ariizumi et al., 2011). Cluster analysis revealed that SlVPE3 and SlVPE5, which are involved in sugar metabolism, did not cluster with other tomato VPE proteases but were rather the most closely related to tobacco (*Nicotiana tabacum*) NtVPE3 protein and sweet pepper CaVPE1b protein (Fig. 1B). This suggests that there might be a class of proteases among the VPE family proteins that specifically regulate

the accumulation of sugars.

Characterization of targeted editing in transgenic plants

The transformation system of *SlINVINH1* gene knock-out finally yielded 14 T_0 generation lines positive for the Cas9 gene. The genomic DNA was extracted from the leaves of each line, following which PCR using primers designed for the target region was carried out to amplify the mutant region fragments, and each clone of the amplified products were sequenced and analyzed to obtain information on mutations in the target region of the *INVINH1* gene. The sequencing results demonstrated that among the 14 lines that were positive for Cas9, only 6 had a mutation in the target sequence of *SlINVINH1* gene (probability of mutation = 42.86%). Among the mutants, one line was a homozygous mutant, two were biallelic mutants, one was a heterozygous mutant, and two were chimeric mutants (Fig. 2C, D; Table 1). The detailed mutations in the target sequence were mainly the deletions of single or multiple nucleotides (Fig. 2C).

The transformation system of the *SlVPE5* gene knock-out yielded 13 T_0 generation lines that were positive for the Cas9 gene. The results of mutation detection revealed that among the 13 Cas9-positive lines, 8 lines had mutations in the target sequence of the *SlVPE5* gene (probability of mutation = 61.54%). Among the mutants, one line was a homozygous mutant, another line was a biallelic mutant, five lines were heterozygous mutants, and one line was a chimeric mutant (Fig. 2C, D; Table 1). The detailed mutations in the target region were mainly the deletion and replacement of single or multiple nucleotides (Fig. 2C).

Further analysis revealed that the mutation types of the *SlINVINH1* gene were mainly biallelic mutations and chimeras, while the mutation type of the *SlVPE5* gene was mainly heterozygotic (Fig. 2C, D; Table 1). In addition, at target-1 site of the *SlINVINH1* gene, there are 14 mutations (2 mutations of homozygous mutant of alleles), at its target-2 site, there are 9 mutations. In the gene *SlVPE5*, at target-1 site, there are 10 mutations, while at the target-2 site, there are 8 mutations (Fig. 2C). This might be related to the promoters of sgRNA; the sgRNA of target-1 was initiated by the U6 promoter of *Arabidopsis*, while the sgRNA of target-2 was initiated by the U6 promoter of tomato. In this study, we observed that the efficiency of the *Arabidopsis* U6 promoter was higher

than that of the tomato U6 promoter.

CRISPR/Cas9-~~mediared mutants exhibited increase the soluble sugar and TSS content~~

The T_0 mutant line, obtained as described in the above section, was selected, and the content of soluble sugar and TSS in the fruit was determined after maturation, each line with three replications. Among the mutants of the *SlINVINH1* gene, four lines, INH-1, INH-2, INH-3, and INH-4 (Fig. 2C), were selected (the lines INH-5 and INH-6 were not selected as they were chimeras and displayed an extremely low probability of passing the mutations on to the next generation). ~~Each line with three replications,~~ It was observed that the contents of both glucose and fructose in the fruits of the lines INH-1, INH-2, and INH-4 were significantly increased, compared to the wild-type (INH-1 showed 40.19% increase in glucose and 42.42% increase in fructose; INH-2 showed 36.39% increase in glucose and 35.69% increase in fructose; INH-4 showed 40.82% increase in glucose and 42.76% increase in fructose) (Fig. 3A; Table S2). In addition, the TSS content also showed a significant increase (31.90% in INH-1, 30.17% in INH-2, 32.76% in INH-4) (Fig. 3A; Table S2), indicating that the *SlINVINH1* gene mainly regulated the accumulation of glucose and fructose in fruits. However, the content of soluble sugars in the fruit of heterzygous INH-3 did not increase significantly compared to the wild-type (Fig. 3A; Table S2), which could be due to the recessive function of the mutated *slinvinh1* gene.

Among the T_0 lines that carried the mutations of the *SlVPE5* gene, three lines, VPE-1, VPE-2, and VPE-7, were selected (the line VPE-8 was not selected as it was chimera and showed an extremely low probability of passing the mutations to the next generation. Also, among the five heterzygous, only one line, namely VPE-2, was selected as a representative for testing). The test revealed that glucose and fructose content in the lines fruits of VPE-1 and VPE-7 also increased significantly (glucose increase 35.20% in VPE-1, and increase 35.83% in VPE-7; fructose increase 37.33% in VPE-1, and increase 43.00% in VPE-7), and the TSS content also increased significantly (30.63% in VPE-1, 32.43% in VPE-7) (Fig. 3B; Table S2), indicating that the *SlVPE5* gene also mainly regulated the accumulation of glucose and fructose in fruits. However, the soluble sugar content in the fruits of heterzygous INH-2 did not increase significantly compared to the

wild-type (Fig. 3B; Table S2), which might also be caused by the recessive gene characteristics of the mutant *slvpe5* gene.

The double homozygous mutants further increased the soluble sugar and TSS content

The T_0 generation homozygous mutant lines INH-4 and VPE-7 were ~~crossed pollination~~ to generate the F_1 generation. The F_2 generation was obtained by self-pollination of the F_1 generation, and 515 well-grown lines were obtained after sowing the F_2 generation seeds. ~~The genotype was screened~~, and 32 lines with homozygous mutated sites (genotype: *inh/inh-vpe/vpe*) of *SlINVINH1* and *SlVPE5* were obtained from the 515 lines. By further screening for the exogenous Cas9 genes among the 32 lines, two lines without the exogenous Cas9 gene were identified. The two ~~individual~~ lines were numbered as F_2–1 and F_2–2.

The ~~contents of~~ soluble sugar and TSS in the fruits of F_2–1 and F_2–2 lines were determined after maturation, each line with three replications. ~~It was observed that~~ the content~~s~~ of glucose and fructose in F_2–1 and F_2–2 fruits ~~were~~ significantly increased compared to the wild-type (In F_2–1, glucose increased by 64.86%, and fructose increased by 68.40%, while in F_2–2, glucose increased by 67.41%, and fructose increased by 69.44%) (Fig. 4; Table S3). The TSS content also showed a significant increase (55.17% in F_2–1 and 62.07% in F_2–2) (Fig. 4; Table S3).

As described earlier, we observed that both the *SlINVINH1* and *SlVPE5* gene mainly regulated the accumulation of glucose and fructose in the fruit. In addition, the content of both glucose and fructose, was significantly synergistic increased in the F_2–1 and F_2–2 fruits, and the TSS content was also significantly higher than the single locus-mutated homozygote. This indicated that the *SlINVINH1* and *SlVPE5* genes displayed a synergistic effect in regulating the content of soluble sugar.

Dynamic variation pattern of the fruit development and coloring

Because the *SlINVINH1* is an invertase gene, loss of invertase function may delay fruit ripening. On the other hand, the loss of *SlVPE5* gene function~~s~~ may also cause changes in the other commercial traits ~~(such as the size of the fruit, the color of the fruit) of tomato fruits~~. This study monitored the ~~entire process of development~~ of two ~~individual~~ lines (F_2–1 and F_2–2), starting from

293 flowering to fruit ripening, in real time. The results indicated that tomatoes of the F_2–1 and F_2–2
294 lines were similar to the wild-type tomato fruits (including both shape and size of the fruits),
295 starting from flowering to the physiological fruit expansion stage. However, during the breaker
296 stage and the beginning of the ripping stage, fruits of the F_2–1 and F_2–2 lines were significantly
297 delayed the break stage compared to the wild-type fruits, although they were fully ripened at the
298 same time as wild-type fruits (Fig. 5A). This indicated that although the knock-out of the
299 *SlINVINH1* and *SlVPE5* genes did delay the break stage and the color change of tomato fruits, it
300 neither affected the ripening and harvesting time of the fruits nor the color of fruits after ripening
301 (Fig. 5B).

302 # DISCUSSION

303 **The effects of SlINVINH1 and SlVPE5 in increasing the soluble sugar and the TSS content**
304 **of tomato**

305 We observed that the both genes *SlINVINH1* and *SlVPE5* mainly regulated the accumulation of
306 glucose and fructose in fruits (Fig. 3A, 3B; Fig. 4), confirming the findings of previous research
307 (Jin, Ni & Ruan, 2009; Ariizumi et al., 2011; Xu et al., 2017). In addition, there was also previous
308 research finding that *SlVPE5* gene also regulates the accumulation of sucrose in fruits (Wang et
309 al., 2016). However, in this study, no sucrose content was detected in mature fruits (detection
310 threshold was >= 0.2 mg/g fw), no matter in T_0 or F_2 lines. This may be because sucrose content
311 in mature fruits is very low and sucrose contribution to the TSS of mature fruits is very small
312 (Tieman et al., 2017). In homozygotes with the knock-out of both loci, both of tested sugars were
313 significantly increased in the fruit, and the TSS content was also significantly higher than that in
314 single locus-mutated homozygotes, indicating that there was a synergistic effect of *SlINVINH1*
315 and *SlVPE5* genes in regulating the content of soluble sugar. This finding is crucial for the
316 improvement of quantitative genetic traits of soluble sugar.

317 **The knock-out efficiency of different genes and different target sites**

318 The knock-out efficiency of both genes was observed to be different. In the knock-out experiment

on *SlINVINH1*, 6 lines with the successful knock-out of the target gene were obtained from 14 positive lines, and the knock-out efficiency was 42.86%, while in the knock-out experiment on *SlVPE5*, 8 lines with the successful knock-out of the target gene were obtained from 13 positive lines, with a knock-out efficiency of 61.54%. Furthermore, the mutation types of the *SlINVINH1* gene were mainly biallelic mutations and chimeras, while the mutation types of the *SlVPE5* gene were mainly heterozygous (Fig. 2C, D; Table 1), which may be linked to the different types and structures of the genes (Pan et al., 2016; Li et al., 2018).

The sgRNA initiated by the U6 promoter of *Arabidopsis thaliana* showed 14 mutations at the target-1 site of the *SlINVINH1* gene and 10 mutations at the target-1 site of the *SlVPE5* gene, while the sgRNA initiated by the U6 promoter of tomato showed 9 mutations in the target-2 site of the *SlINVINH1* gene and 8 mutations in the target-2 site of the *SlVPE5* gene. Therefore, the efficiency of *Arabidopsis* U6 promoter was observed to be higher than that of the tomato U6 promoter at different target sites of the two genes. Based on these findings, we propose that the use of different promoters of sgRNA may also cause differences in the rate of mutation, which is consistent with the findings of previous studies (Ma et al., 2015; Čermák et al., 2017; Shao et al., 2020).

The methodology used in this study can effectively improve the commercial traits of tomato

In this study, two homozygous lines (F_2–1 and F_2–2) with mutations in both the functional gene loci were obtained, and the exogenous Cas9 was not included, due to which the content of both soluble sugar and TSS in tomato fruits were significantly increased (Fig. 4), thus facilitating the rapid improvement of the commercial traits of tomatoes. This study confirmed the feasibility of target gene editing by the transfer of Cas9 and sgRNA, and the subsequent application of conventional methods such as hybridization and inbreeding. On the one hand, two mutation sites could be brought together, while on the other, the mutation site could be made homozygous, and the exogenous gene Cas9 could be eradicated. This method greatly shortened the breeding cycle during the process of improving tomato varieties, and completely eliminated various risk factors associated with the exogenous genes. This technical system will improve technological innovation and progress in tomato breeding, and has tremendous potential in a variety of applications.

CONCLUSIONS

The purpose of this study was to identify two tomato genes (*SlINVINH1* and *SlVPE5*) that inhibited the accumulation of soluble sugar in tomato fruits, and then to obtain the knock-out lines of two genes by CRISPR/Cas9, respectively. Therewith, the aggregated lines with both CRISPR-*invinh1* and CRISPR-*vpe5* were gained by hybridization and self-pollinating. Compared to the wild-type tomato lines, the glucose, fructose and TSS contents of CRISPR-*invinh1* or CRISPR-*vpe5* were significantly increased. In addition, the levels of glucose, fructose and TSS showed a further improved in the lines with CRISPR-*invinh1* and CRISPR-*vpe5* simultaneously than that of the single gene knock-out lines, which indicated that the two genes had a synergistic effect in increasing the content of these soluble sugars. Thus, knock-out the *SlINVINH1* and *SlVPE5* could effectively increase the content of soluble sugars in tomato and might provide an important theoretical guidance and practical basis for improving the flavor of tomato fruits and processing quality.

Supplemental Information

Table S1. Primers for detection of target region.

Table S2. The content of soluble sugar and TSS in the red fruit of T_0 mutant lines.

Table S3. The content of soluble sugar and TSS in the red fruit of F_2 lines.

REFERENCES

Ariizumi T, Higuchi K, Arakaki S, Sano T, Asamizu E, Ezura H. 2011. Genetic suppression analysis in novel vacuolar processing enzymes reveals their roles in controlling sugar accumulation in tomato fruits. *Journal of Experimental Botany*. DOI: 10.1093/jxb/erq451.

Baxter CJ, Carrari F, Bauke A, Overy S, Hill SA, Quick PW, Fernie AR, Sweetlove LJ. 2005. Fruit carbohydrate metabolism in an introgression line of tomato with increased fruit soluble solids. *Plant and Cell Physiology*. DOI: 10.1093/pcp/pci040.

Beckles DM. 2012. Factors affecting the postharvest soluble solids and sugar content of tomato (Solanum lycopersicum L.) fruit. *Postharvest Biology and Technology*. DOI: 10.1016/j.postharvbio.2011.05.016.

Beckles DM, Hong N, Stamova L, Luengwilai K. 2012. Biochemical factors contributing to tomato fruit sugar

content: A review. *Fruits*. DOI: 10.1051/fruits/2011066.

Čermák T, Curtin SJ, Gil-Humanes J, Čegan R, Kono TJY, Konečná E, Belanto JJ, Starker CG, Mathre JW, Greenstein RL, Voytas DF. 2017. A multipurpose toolkit to enable advanced genome engineering in plants. *Plant Cell*. DOI: 10.1105/tpc.16.00922.

Gur A, Zamir D. 2015. Mendelizing all components of a pyramid of three yield QTL in Tomato. *Frontiers in Plant Science*. DOI: 10.3389/fpls.2015.01096.

Hara-Nishimura I, Takeuchi Y, Nishimura M. 1993. Molecular characterization of a vacuolar processing enzyme related to a putative cysteine proteinase of Schistosoma mansoni. *Plant Cell*. DOI: 10.1105/tpc.5.11.1651.

Jin Y, Ni DA, Ruan YL. 2009. Posttranslational elevation of cell wall invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose Level. *Plant Cell*. DOI: 10.1105/tpc.108.063719.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular biology and evolution*. DOI: 10.1093/molbev/msw054.

Li Y, Lu Y, Li L, Chu Z, Zhang H, Li H, Fernie AR, Ouyang B. 2019. Impairment of hormone pathways results in a general disturbance of fruit primary metabolism in tomato. *Food Chemistry*. DOI: 10.1016/j.foodchem.2018.08.026.

Li X, Wang Y, Chen S, Tian H, Fu D, Zhu B, Luo Y, Zhu H. 2018. Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. *Frontiers in Plant Science*. DOI: 10.3389/fpls.2018.00559.

Lupi ACD, Lira BS, Gramegna G, Trench B, Alves FRR, Demarco D, Peres LEP, Purgatto E, Freschi L, Rossi M. 2019. Solanum lycopersicum GOLDEN 2-LIKE 2 transcription factor affects fruit quality in a light- and auxin-dependent manner. *PLoS ONE*. DOI: 10.1371/journal.pone.0212224.

Ma X, Zhang Y, Turečková V, Xue GP, Fernie AR, Mueller-Roeber B, Balazadeh S. 2018. The NAC transcription factor SLNAP2 regulates leaf senescence and fruit yield in tomato. *Plant Physiology*. DOI: 10.1104/pp.18.00292.

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, Xie Y, Shen R, Chen S, Wang Z, Chen Y, Guo J, Chen L, Zhao X, Dong Z, Liu YG. 2015. A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Molecular Plant* 8:1274–1284. DOI: 10.1016/j.molp.2015.04.007.

Pan C, Ye L, Qin L, Liu X, He Y, Wang J, Chen L, Lu G. 2016. CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Scientific reports* 6:24765. DOI: 10.1038/srep24765.

Patrick JW, Botha FC, Birch RG. 2013. Metabolic engineering of sugars and simple sugar derivatives in plants. *Plant Biotechnology Journal*. DOI: 10.1111/pbi.12002.

Petreikov M, Yeselson L, Shen S, Levin I, Schaffer AA, Efrati A, Bar M. 2009. Carbohydrate balance and accumulation during development of near-isogenic tomato lines differing in the AGPase-L1 allele. *Journal of the American Society for Horticultural Science*. DOI: 10.21273/jashs.134.1.134.

Powell ALT, Nguyen C V., Hill T, Cheng KLL, Figueroa-Balderas R, Aktas H, Ashrafi H, Pons C, Fernández-Muñoz R, Vicente A, Lopez-Baltazar J, Barry CS, Liu Y, Chetelat R, Granell A, Van Deynze A, Giovannoni JJ, Bennett AB. 2012. Uniform ripening encodes a Golden 2-like transcription factor regulating tomato fruit chloroplast development. *Science*. DOI: 10.1126/science.1222218.

Qin G, Zhu Z, Wang W, Cai J, Chen Y, Li L, Tian S. 2016. A tomato vacuolar invertase inhibitor mediates sucrose

metabolism and influences fruit ripening. *Plant Physiology*. DOI: 10.1104/pp.16.01269.

Quan J, Tian J. 2009. Circular polymerase extension cloning of complex gene libraries and pathways. *PLoS ONE*. DOI: 10.1371/journal.pone.0006441.

Rausch T, Greiner S. 2004. Plant protein inhibitors of invertases. *Biochimica et Biophysica Acta - Proteins and Proteomics*. DOI: 10.1016/j.bbapap.2003.09.017.

Reca IB, Brutus A, D'Avino R, Villard C, Bellincampi D, Giardina T. 2008. Molecular cloning, expression and characterization of a novel apoplastic invertase inhibitor from tomato (Solanum lycopersicum) and its use to purify a vacuolar invertase. *Biochimie*. DOI: 10.1016/j.biochi.2008.04.019.

Ruan YL, Jin Y, Huang J. 2009. Capping invertase activity by its inhibitor: Roles and implications in sugar signaling, carbon allocation, senescence and evolution. *Plant Signaling and Behavior*. DOI: 10.4161/psb.4.10.9667.

Shao X, Wu S, Dou T, Zhu H, Hu C, Huo H, He W, Deng G, Sheng O, Bi F, Gao H, Dong T, Li C, Yang Q, Yi G. 2020. Using CRISPR/Cas9 genome editing system to create MaGA20ox2 gene-modified semi-dwarf banana. *Plant Biotechnology Journal*. DOI: 10.1111/pbi.13216.

Tieman D, Zhu G, Resende MFR, Lin T, Nguyen C, Bies D, Rambla JL, Beltran KSO, Taylor M, Zhang B, Ikeda H, Liu Z, Fisher J, Zemach I, Monforte A, Zamir D, Granell A, Kirst M, Huang S, Klee H. 2017. A chemical genetic roadmap to improved tomato flavor. *Science (New York, N.Y.)*. DOI: 10.1126/science.aal1556.

Vallarino JG, Yeats TH, Maximova E, Rose JK, Fernie AR, Osorio S. 2017. Postharvest changes in LIN5-down-regulated plants suggest a role for sugar deficiency in cuticle metabolism during ripening. *Phytochemistry*. DOI: 10.1016/j.phytochem.2017.06.007.

Wang N, Duhita N, Ariizumi T, Ezura H. 2016. Involvement of vacuolar processing enzyme SlVPE5 in post-transcriptional process of invertase in sucrose accumulation in tomato. *Plant Physiology and Biochemistry*. DOI: 10.1016/j.plaphy.2016.06.037.

Wu L, Birch RG. 2007. Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. *Plant Biotechnology Journal*. DOI: 10.1111/j.1467-7652.2006.00224.x.

Xu X xia, Hu Q, Yang W nian, Jin Y. 2017. The roles of call wall invertase inhibitor in regulating chilling tolerance in tomato. *BMC Plant Biology*. DOI: 10.1186/s12870-017-1145-9.

Yu QH, Wang B, Li N, Tang Y, Yang S, Yang T, Xu J, Guo C, Yan P, Wang Q, Asmutola P. 2017. CRISPR/Cas9-induced Targeted Mutagenesis and Gene Replacement to Generate Long-shelf Life Tomato Lines. *Scientific Reports*. DOI: 10.1038/s41598-017-12262-1.

Zanor MI, Osorio S, Nunes-Nesi A, Carrari F, Lohse M, Usadel B, Kühn C, Bleiss W, Giavalisco P, Willmitzer L, Sulpice R, Zhou YH, Fernie AR. 2009. RNA interference of LIN5 in tomato confirms its role in controlling brix content, uncovers the influence of sugars on the levels of fruit hormones, and demonstrates the importance of sucrose cleavage for normal fruit development and fertility. *Plant Physiology*. DOI: 10.1104/pp.109.136598.

455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475

Figure Legends

Figure 1 Phylogenetic analysis of INVINH and VPE proteins in plants. (A) Phylogenetic tree of INVINH. (B) Phylogenetic tree of VPE. At, *Arabidopsis thaliana*; Ca, *Capsicum annuum*; Cs, *Citrus sinensis*; Gm, *Glycine max*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; St, *Solanum tuberosum*; Vv, *Vitis vinifera*; Zm, *Zea mays*. The size bar shows the estimated evolutionary distance.

Figure 2 Target genes editing by CRISPR/Cas9. (A) The schematic description of CRISPR/Cas9-sgRNA expression cassette. SpCas9 is controlled by a CaMV 35S promoter, the first sgRNA is expressed by the AtU6 promoter, the second sgRNA is expressed by the SlU6 promoter. (B) Four target sites were designed in two target genes. Straight blue lines and

rectangular orange boxes are the introns and exons of the target genes, respectively. (C) The sequenced targeted InDel mutation of two target gene. 26 clones of the each PCR amplicon were picked and sequenced. “WT” means Wild-type, “Bi” means Biallele, “He” means Heterozygote, “Ho” means Homozygote, “Ch” means Chimera. (D) Specific types of each target gene in T_0 lines. Green, orange, purple, and blue represent homozygous, biallelic, heterozygous, and chimeric mutations, respectively.

Figure 3 Determination of the soluble sugar and TSS content in red fruit from different T_0 lines and WT. (A) The contents of the soluble sugar and TSS of red fruit in four different T_0 lines of editing *SlINVINH1* gene and WT. (B) The contents of the soluble sugar and TSS of red fruit in three different T_0 lines of editing *SlVPE5* gene and WT. The contents of glucose and fructose is measured in mg/g in fruit weigh, and the TSS is measured in Brix (%). *($P < 0.05$, Student’s t-test, n = 3) indicate statistically significant differences between T_0 mutant lines and wild-type.

Figure 4 Determination of the soluble sugar and TSS content in red fruit from the WT and the two F_2 lines of double homozygous mutants of edited *SlINVINH1* and *SlVPE5* genes. The contents of glucose and fructose is measured in mg/g in fruit weigh, and the TSS is measured in Brix (%). **($P < 0.01$, Student’s t-test, n = 3) indicate statistically highly significant differences between F_2 lines and wild-type.

Figure 5 Phenotypic detection of the WT fruits and the two F_2 lines fruits. (A) Developmental series of WT fruits (up) and F_2 fruits (down), “dpa” is days post anthesis. (B) Comparison of bisected fruit at the ripening stage. Bars = 1 cm.

Figure 1

Phylogenetic analysis of INVINH and VPE proteins in plants

Phylogenetic analysis of INVINH and VPE proteins in plants. (A) Phylogenetic tree of INVINH. (B) Phylogenetic tree of VPE. At, *Arabidopsis thaliana*; Ca, *Capsicum annuum* ; Cs, *Citrus sinensis*; Gm, *Glycine max*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; St, *Solanum tuberosum*; Vv, *Vitis vinifera*; Zm, *Zea mays*. The size bar shows the estimated evolutionary distance.

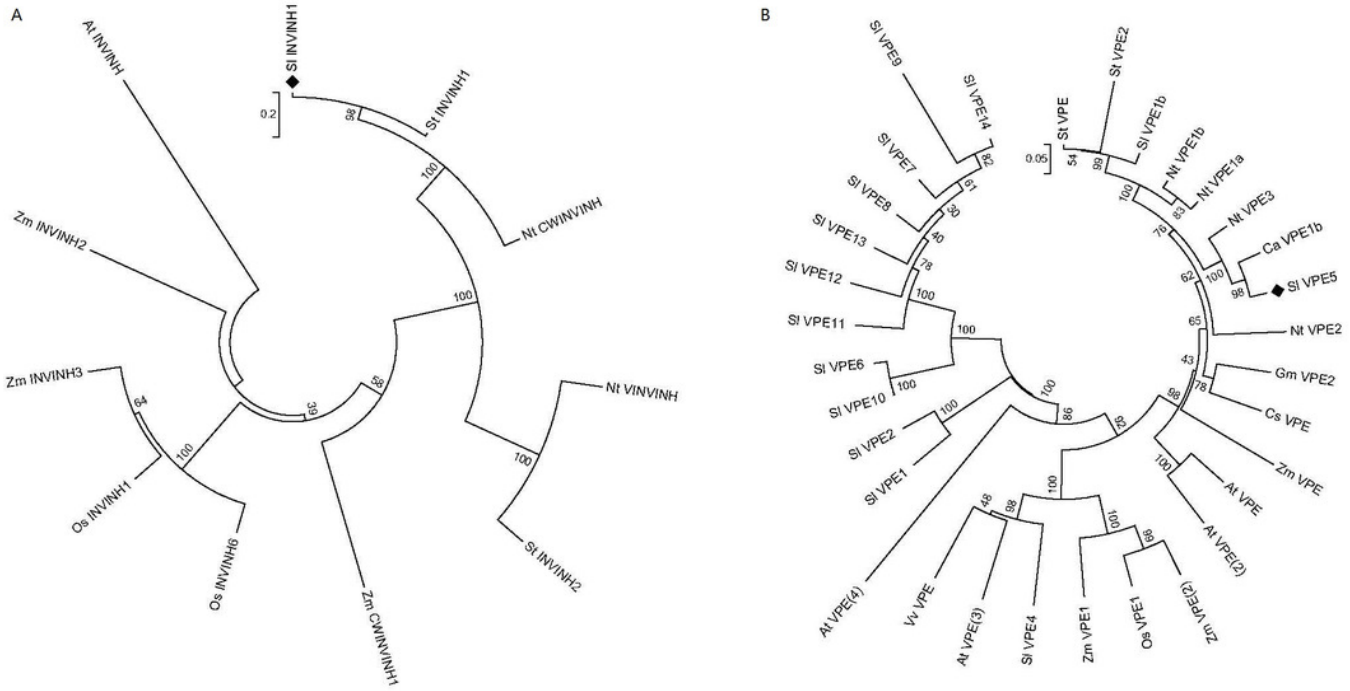

Figure 2

Target genes editing by CRISPR/Cas9

Target genes editing by CRISPR/Cas9. (A) The schematic description of CRISPR/Cas9-sgRNA expression cassette. SpCas9 is controlled by a CaMV 35S promoter, the first sgRNA is expressed by the AtU6 promoter, the second sgRNA is expressed by the SlU6 promoter. (B) Four target sites were designed in two target genes. Straight blue lines and rectangular orange boxes are the introns and exons of the target genes, respectively. (C) The sequenced targeted InDel mutation of two target gene. 26 clones of the each PCR amplicon were picked and sequenced. “WT” means Wild-type, “Bi” means Biallele, “He” means Heterozygote, “Ho” means Homozygote, “Ch” means Chimera. (D) Specific types of each target gene in T_0 lines. Green, orange, purple, and blue represent homozygous, biallelic, heterozygous, and chimeric mutations, respectively.

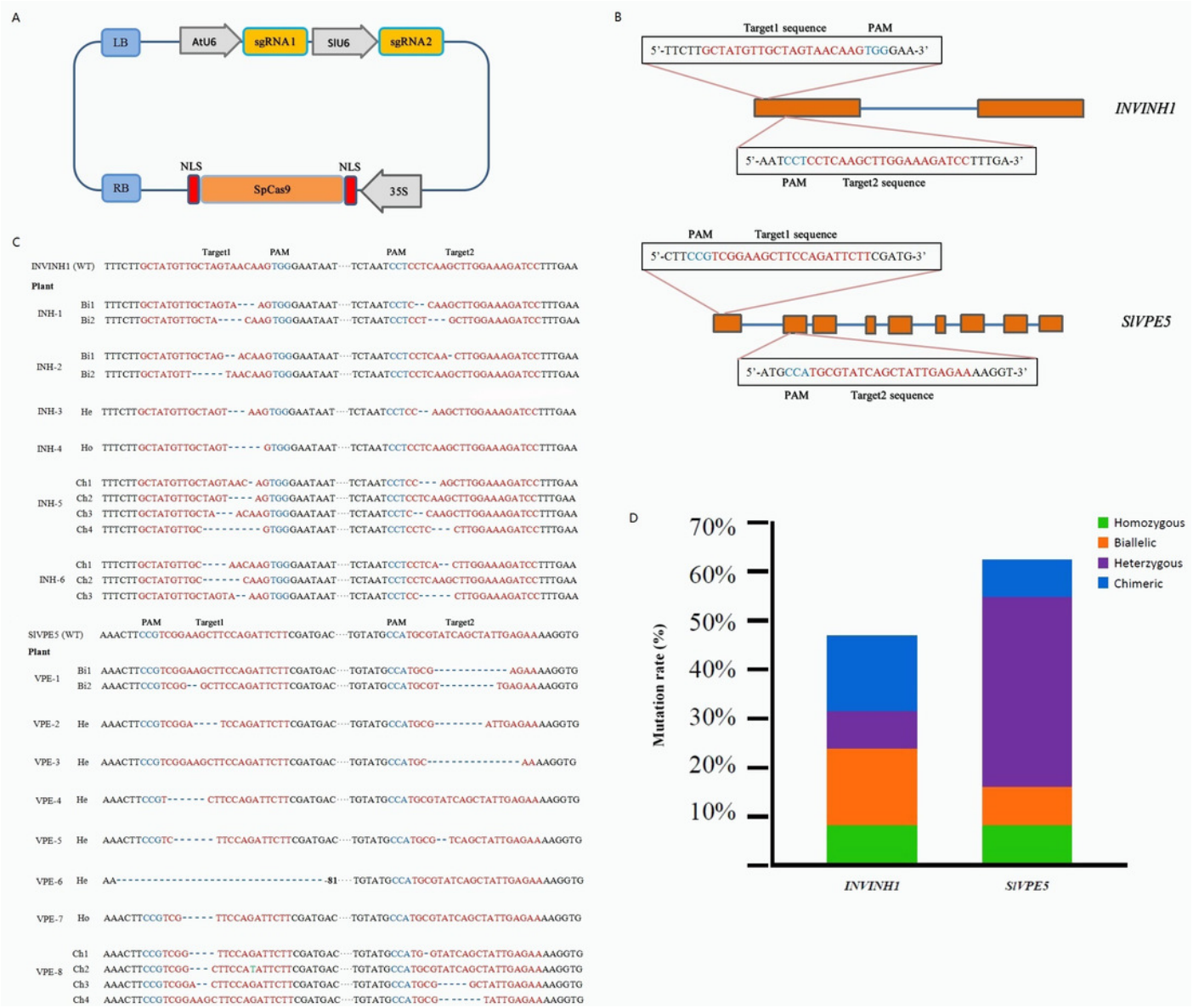
A
LB
AtU6
sgRNA1
SlU6
sgRNA2
RB
NLS
SpCas9
NLS
35S
B
Target1 sequence
PAM
5'-TTCTTGCTATGTTGCTAGTAACAAGTGGGAA-3'
INVINH1
5'-AATCCTCCTCAAGCTTGGAAAGATCCTTTGA-3'
PAM
Target2 sequence
PAM
Target1 sequence
5'-CTTCCGTCGGAAGCTTCCAGATTCTTCGATG-3'
SlVPE5
5'-ATGCCATGCGTATCAGCTATTGAGAAAAGGT-3'
PAM
Target2 sequence
C
Target1
PAM
PAM
Target2
INVINH1 (WT) TTTCTTGCTATGTTGCTAGTAACAAGTGGGAATAAT···TCTAATCCTCCTCAAGCTTGGAAAGATCCTTTGAA
Plant
INH-1
Bi1 TTTCTTGCTATGTTGCTAGTA---AGTGGGAATAAT···TCTAATCCTC--CAAGCTTGGAAAGATCCTTTGAA
Bi2 TTTCTTGCTATGTTGCTA----CAAGTGGGAATAAT···TCTAATCCTCCT---GCTTGGAAAGATCCTTTGAA
INH-2
Bi1 TTTCTTGCTATGTTGCTAG--ACAAGTGGGAATAAT···TCTAATCCTCCTCAA-CTTGGAAAGATCCTTTGAA
Bi2 TTTCTTGCTATGTT-----TAACAAGTGGGAATAAT···TCTAATCCTCCTCAAGCTTGGAAAGATCCTTTGAA
INH-3
He TTTCTTGCTATGTTGCTAGT---AAGTGGGAATAAT···TCTAATCCTCC--AAGCTTGGAAAGATCCTTTGAA
INH-4
Ho TTTCTTGCTATGTTGCTAGT-----GTGGGAATAAT···TCTAATCCTCCTCAAGCTTGGAAAGATCCTTTGAA
INH-5
Ch1 TTTCTTGCTATGTTGCTAGTAAC-AGTGGGAATAAT···TCTAATCCTCC---AGCTTGGAAAGATCCTTTGAA
Ch2 TTTCTTGCTATGTTGCTAGT----AGTGGGAATAAT···TCTAATCCTCCTCAAGCTTGGAAAGATCCTTTGAA
Ch3 TTTCTTGCTATGTTGCTA---ACAAGTGGGAATAAT···TCTAATCCTC--CAAGCTTGGAAAGATCCTTTGAA
Ch4 TTTCTTGCTATGTTGC---------GTGGGAATAAT···TCTAATCCTCCTC---CTTGGAAAGATCCTTTGAA
INH-6
Ch1 TTTCTTGCTATGTTGC----AACAAGTGGGAATAAT···TCTAATCCTCCTCA--CTTGGAAAGATCCTTTGAA
Ch2 TTTCTTGCTATGTTGC------CAAGTGGGAATAAT···TCTAATCCTCCTCAAGCTTGGAAAGATCCTTTGAA
Ch3 TTTCTTGCTATGTTGCTAGTA--AAGTGGGAATAAT···TCTAATCCTCC-----CTTGGAAAGATCCTTTGAA
PAM
Target1
PAM
Target2
SlVPE5 (WT) AAACTTCCGTCGGAAGCTTCCAGATTCTTCGATGAC···TGTATGCCATGCGTATCAGCTATTGAGAAAAGGTG
Plant
VPE-1
Bi1 AAACTTCCGTCGGAAGCTTCCAGATTCTTCGATGAC···TGTATGCCATGCG----------AGAAAAGGTG
Bi2 AAACTTCCGTCGG--GCTTCCAGATTCTTCGATGAC···TGTATGCCATGCGT---------TGAGAAAAGGTG
VPE-2
He AAACTTCCGTCGGA----TCCAGATTCTTCGATGAC···TGTATGCCATGCG--------ATTGAGAAAAGGTG
VPE-3
He AAACTTCCGTCGGAAGCTTCCAGATTCTTCGATGAC···TGTATGCCATGC----------AAAAGGTG
VPE-4
He AAACTTCCGT------CTTCCAGATTCTTCGATGAC···TGTATGCCATGCGTATCAGCTATTGAGAAAAGGTG
VPE-5
He AAACTTCCGTC------TTCCAGATTCTTCGATGAC···TGTATGCCATGCG--TCAGCTATTGAGAAAAGGTG
VPE-6
He AA----------81···TGTATGCCATGCGTATCAGCTATTGAGAAAAGGTG
VPE-7
Ho AAACTTCCGTCG-----TTCCAGATTCTTCGATGAC···TGTATGCCATGCGTATCAGCTATTGAGAAAAGGTG
VPE-8
Ch1 AAACTTCCGTCGG----TTCCAGATTCTTCGATGAC···TGTATGCCATG-GTATCAGCTATTGAGAAAAGGTG
Ch2 AAACTTCCGTCGG---CTTCCATATTCTTCGATGAC···TGTATGCCATGCGTATCAGCTATTGAGAAAAGGTG
Ch3 AAACTTCCGTCGGA--CTTCCAGATTCTTCGATGAC···TGTATGCCATGCG-----GCTATTGAGAAAAGGTG
Ch4 AAACTTCCGTCGGAAGCTTCCAGATTCTTCGATGAC···TGTATGCCATGCG-------TATTGAGAAAAGGTG
D
Mutation rate (%)
70%
60%
50%
40%
30%
20%
10%
Homozygous
Biallelic
Heterzygous
Chimeric
INVINH1
SlVPE5

Figure 3

Determination of the soluble sugar and TSS content in red fruit from different T_0 lines and WT

Determination of the soluble sugar and TSS content in red fruit from different T_0 lines and WT. (A) The contents of the soluble sugar and TSS of red fruit in four different T_0 lines of editing *SlINVINH1* gene and WT. (B) The contents of the soluble sugar and TSS of red fruit in three different T_0 lines of editing *SlVPE5* gene and WT. The contents of glucose and fructose is measured in mg/g in fruit weigh, and the TSS is measured in Brix (%). *($P < 0.05$, Student's t-test, n = 3) indicate statistically significant differences between T_0 mutant lines and wild-type.

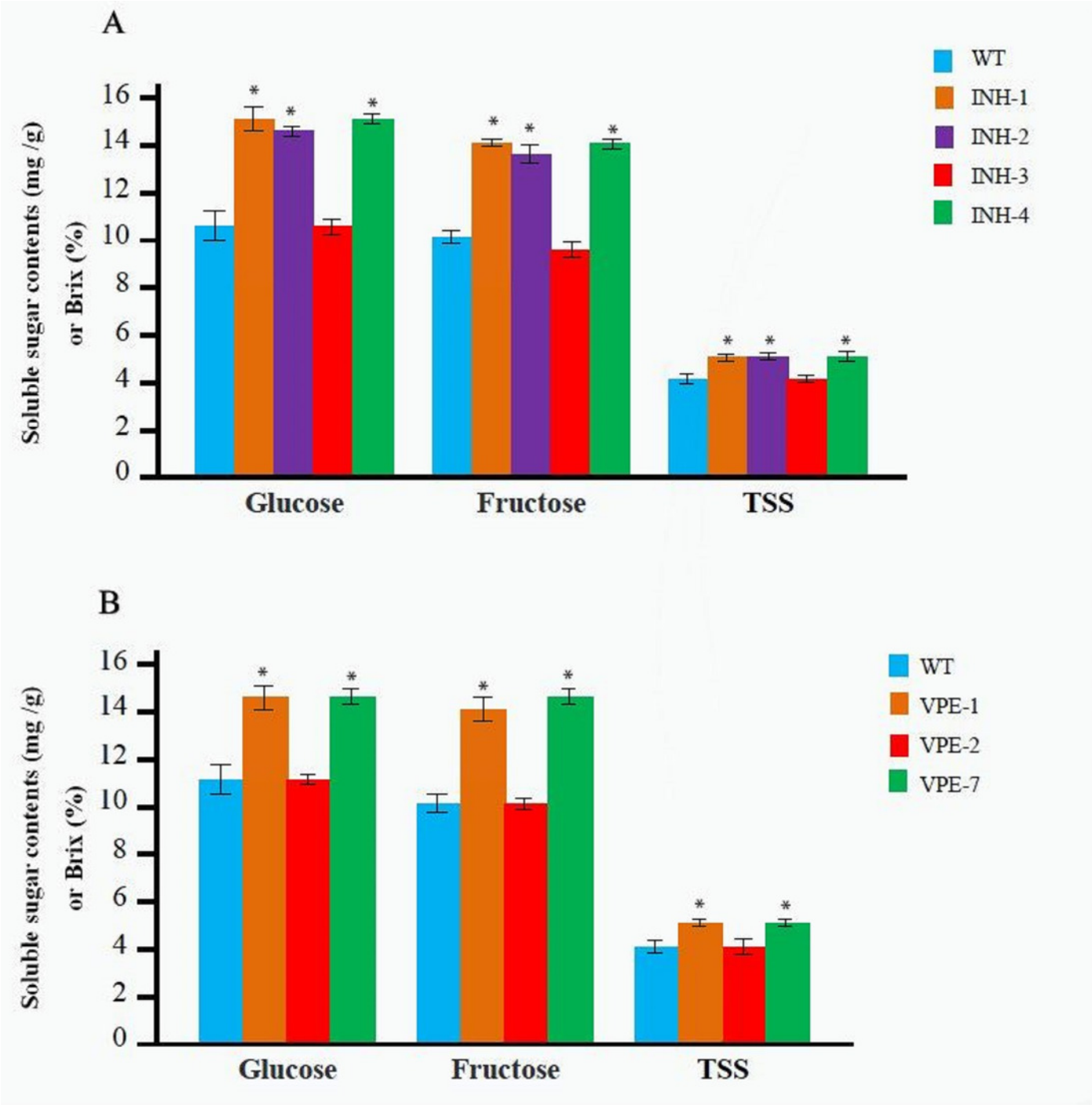
A
Soluble sugar contents (mg /g) or Brix (%)
0
2
4
6
8
10
12
14
16
Glucose
Fructose
TSS
WT
INH-1
INH-2
INH-3
INH-4
B
Soluble sugar contents (mg /g) or Brix (%)
0
2
4
6
8
10
12
14
16
Glucose
Fructose
TSS
WT
VPE-1
VPE-2
VPE-7

Figure 4

Determination of the soluble sugar and TSS content in red fruit from the WT and the two F_2 lines of double homozygous mutants of edited *SlINVINH1* and *SlVPE5* genes

Determination of the soluble sugar and TSS content in red fruit from the WT and the two F_2 lines of double homozygous mutants of edited *SlINVINH1* and *SlVPE5* genes. The contents of glucose and fructose is measured in mg/g in fruit weigh, and the TSS is measured in Brix (%). $^{**}(P < 0.01$, Student's t-test, n = 3) indicate statistically highly significant differences between F_2 lines and wild-type.

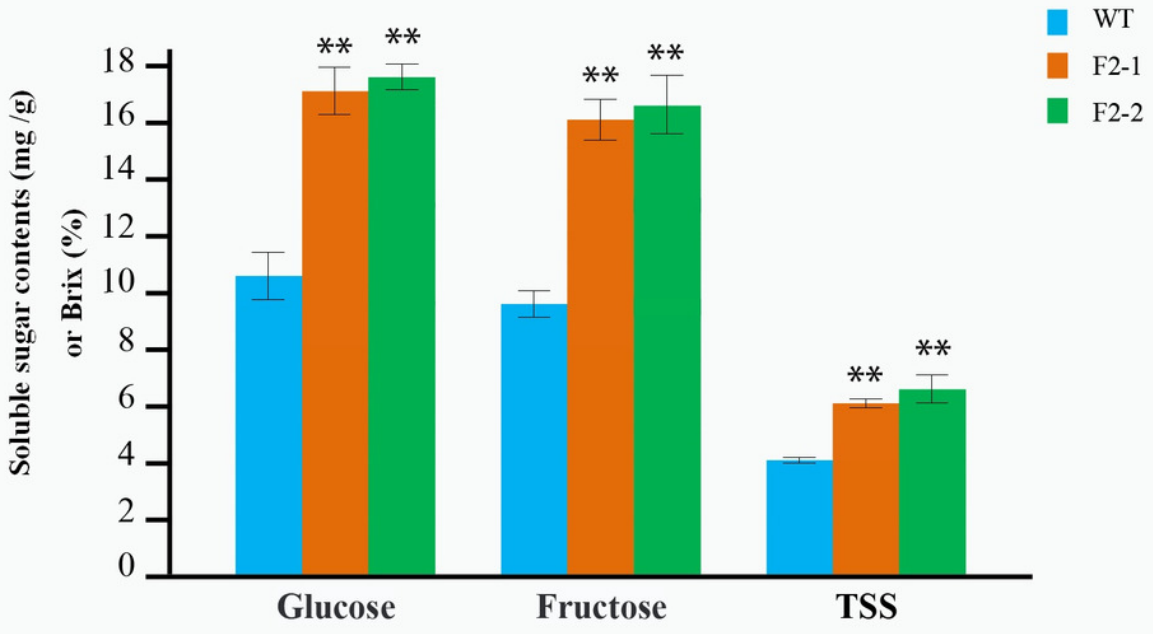

Figure 5

Phenotypic detection of the WT fruits and the two F_2 lines fruits

Phenotypic detection of the WT fruits and the two F_2 lines fruits. (A) Developmental series of WT fruits (up) and F_2 fruits (down), “dpa” is days post anthesis. (B) Comparison of bisected fruit at the ripening stage. Bars = 1 cm.


A
10 dpa
20 dpa
30 dpa
40 dpa
45 dpa
50 dpa
WT
F2
B
WT
F2

Table 1(on next page)

Detected zygosity of T_0 independent transgenic lines

Detected zygosity of T_0 independent transgenic lines of *SlINVINH1* and *SlVPE5*

1 **Table 1. Detected zygosity of T_0 independent transgenic lines of *SlINVINH1* and *SlVPE5***

Target gene	No. of plants examined	Zygosity				
		Homozygote	Biallele	Heterozygote	Chimera	WT
SlINVINH1	14	1(7.14%)	2(14.29%)	1(7.14%)	2(14.29%)	8(57.14%)
SlVPE5	13	1(7.69%)	1(7.69%)	5(38.46%)	1(7.69%)	5(38.46%)

2

3